Chapter 6

Plant Metabolomics in a Changing World: Metabolite Responses to Abiotic Stress Combinations

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Additional information is available at the end of the chapter

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Abstract

Climate change constitutes a real threat to the global landscape. Current climate models predict an increased occurrence of coastal floods associated to sea level rise and long-term droughts associated to changes in the intra- and inter-year rainfall variability. Under natural environmental conditions, plants are routinely exposed to abiotic stresses, and must develop different strategies to cope with this multitude of climate change factors. Mass spectrometry (MS)-based plant metabolomics approaches are finding an increasing number of applications to investigate the molecular and biochemical mechanisms that underlie plant responses to changing environments. These studies provide a promising basis for facilitating our understanding of the plant's flexibility to reconfigure central metabolic pathways (i.e., carbon, nitrogen and energy metabolism) as well as the degree by which plants tolerate and/or are susceptible to a climate change scenario. In this chapter, we will provide an update on the recent MS-based metabolomics strategies to study plant responses to drought, salt and heat stress as well as combinations thereof. We will describe how these stresses activate and coordinate several different signalling pathways, for example, through the synthesis of osmolytes.

Keywords: plant metabolomics, drought stress, salinity stress, heat stress, stress combination, climate change, mass spectrometry

1. Introduction

Climate change can be defined as a statistically significant variation in the weather pattern or in its variability during a long-term period [1]. The causes of climate change have been mainly associated to (i) internal environmental processes and (ii) anthropogenic activities that lead to changes in the chemical composition of the atmosphere [1]. Natural climate variability itself is not enough to explain the unforeseen weather changes in the last decades. In fact,

since the industrial revolution that human-kind activities (e.g., fossil fuel burning) have also contributed to the release of significant amounts of greenhouse gases (GHGs) namely CO_2, CH_4, N_2O as well as fluorinated gases to the atmosphere [1]. Indeed, climate change assessments have reported that the global atmospheric CO_2 concentration has increased from 270 to 401 μL L^{-1} since the industrial revolution, and consequently, the average global temperatures to rise by 0.85°C. Moreover, global warming has been reported to be highly correlated with ocean thermal expansion and loss of glacier mass, which ultimately reflected the observed global mean sea level rise of 0.19 ± 0.02 m over the period 1901–2010 [2]. By the end of the twenty-first century, unmatched climate changes are envisaged with CO_2 concentrations of at least 700 μL L^{-1} and global temperatures are expected to rise at least 4°C. Consequently, higher surface temperatures, longer and frequent heat waves and intense extreme precipitation events are very likely to occur in many regions around the globe. The consequences from climate change cannot be totally avoided, but without additional mitigation efforts beyond those already in place today, warming by the end of the twenty-first century will lead to very high risk of severe and irreversible impacts globally [2].

Extreme climate change events expose plants to stressful environmental conditions that are outside of their physiological limits, and beyond the range by which they are already adapted [3]. Studies aiming at assessing the impact of climate change in plant ecosystems revealed that plant community responses occur at three sequential levels in which (i) climate change immediately impacts plant individuals at the morpho-physiological level, (ii) the community response is affected because of demographic changes in species abundances and (iii) the mortality or loss of species leads to their replacement by novel species within the community [4–6]. Although some studies have contributed to a better understanding of plant ecosystem responses to climate change, this research field is still emerging. A comprehensive discussion on this topic falls outside the scope of this chapter, and detailed information can be found elsewhere [4–13].

Responses by individual plant species to climate change have been indirectly studied through the assessment of the strategies and mechanisms by which they cope with adverse environmental conditions, that is, abiotic stresses. Abiotic stresses in plants comprise a multitude of environmental factors such as water (drought, flooding and submergence), temperature (high and low), light (high and low), radiation (UV-B and UV-A), salinity and nutrients, heavy metals, among others. These environmental (stress)factors negatively affect plant growth and development, and trigger a series of high-complex adaptive responses initiated by stress perception, signal transduction and the activation of many stress-related genes and metabolites [14, 15]. However, under natural environmental conditions, plants are routinely exposed to a combination of different abiotic stresses, and therefore, must develop different strategies to cope with a multitude of environmental factors. The latter gains more relevance under climate change scenarios, and therefore, there has been an increasing interest in understanding the molecular and biochemical mechanisms that underlie plant responses to abiotic stress combinations [16, 17].

Many studies, at both physiological and biochemical levels, have been performed to study plant responses to different stress combinations namely drought, salt, extreme temperatures and biotic stresses. Interestingly, these studies demonstrated that a plant response to a combined stress is unique, and should not be regarded as the sum of the responses from each

applied stress alone. Additionally, when different stresses are combined, they might require synergistic or antagonistic responses that are largely controlled by, sometimes, opposing signalling pathways [16, 17]. In this chapter, we will provide an update on recent studies of plant responses to drought, salt and heat stress as well as combinations thereof. We will describe how these abiotic stress combinations activate and coordinate several different signalling pathways, for example, through the synthesis of osmolytes, in order to ensure plant survival.

2. Metabolomics—a key *omics* tool to study plant responses to abiotic stress

Over the past decade, plant metabolomics has undoubtedly become a powerful research tool to study the biochemical mechanisms underlying plant growth and development in the context of plant metabolite responses to abiotic stress, particularly drought, flooding, salinity and extreme temperatures (heat and cold). In fact, metabolomics itself, together with the other *omics* technologies (genomics, transcriptomics and proteomics) has accelerated our understanding of the complex molecular interactions in biological systems [18–24] (**Figure 1**). Moreover, unlike other *omics* tools, metabolomics has the advantage of not being dependent on the availability of organism-specific genome information for data analysis [25–27]. The main goal of plant metabolomics is to provide a non-biased characterisation of the total metabolite pool of a plant tissue in response to its environment. This metabolite pool includes a wide range of metabolites with diverse physical properties, from ionic inorganic compounds to biochemically derived hydrophilic carbohydrates, organic and amino acids, and a range of hydrophobic lipid-related compounds. Indeed, it is estimated that more than 200,000 different primary and secondary metabolites exist in the plant kingdom over a large dynamic range in concentrations that can vary from femtomolar to millimolar [28]. While, primary metabolites are fundamental for plant growth and development, being highly conserved in their molecular structures and abundances throughout the plant kingdom, secondary metabolites help plants communicating with the environment and widely differ across species. Due to such metabolite diversity, current plant metabolomics studies often combine multiple analytical tools in an effort to acquire more comprehensive metabolite coverage from a complex biological plant sample. One powerful analytical tool is nuclear magnetic resonance (NMR); however, due

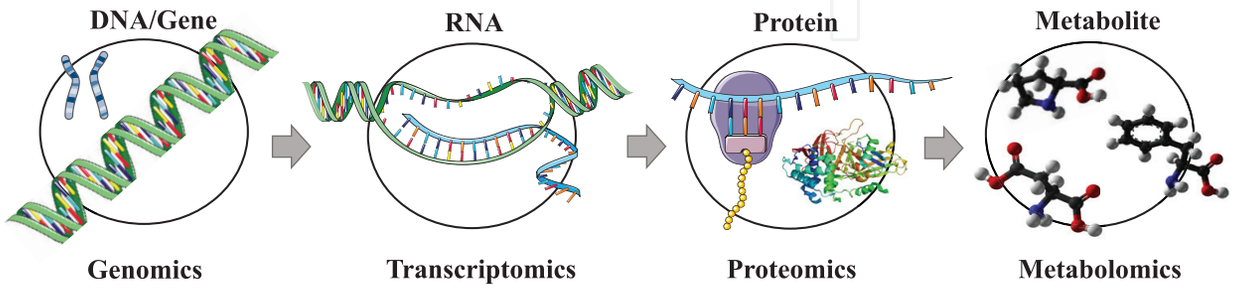

Figure 1. *Omics* technologies have accelerated our understanding of the complex molecular interactions in biological systems.

to its poor sensitivity and poor dynamic range relative to mass spectrometry (MS) [29, 30], MS-based analytical tools are the most widely used in plant metabolomics. Among them, powerful chromatographic techniques such as liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) have been extensively used to obtain comprehensive information of the plant metabolome in a wide range of plant species [24, 31]. Regardless the analytical platform of choice, great attention must be paid to the experimental design. In plant metabolomics, an adequate and well-studied experimental design should address different environmental and experimental variables such as (i) plant tissue harvest, (ii) metabolic quenching and (iii) metabolite extraction methods. In addition, randomisation procedures throughout all the experimental workflow should be taken into account to minimise potential sources of experimental errors [32, 33]. A detailed discussion of sample preparation workflows and MS-based analytical platforms typically used in plant metabolomics experiments can be found elsewhere [24, 34].

3. Plant metabolite responses to individual abiotic stresses

Metabolite responses to individual abiotic stresses such as drought, salinity or heat have been widely studied, and comprehensive reviews on this topic can be found in the literature [24, 31]. In this section, we describe recent applications of MS-based metabolomics approaches to study plant responses to individual abiotic stresses, namely drought, salt and heat stress, highlighting the identification of stress-responsive metabolites that ultimately contribute for the development of plants with enhanced abiotic stress-tolerance.

3.1. Metabolite responses to drought stress

Drought is a well-studied abiotic stress, and one major limiting factor in agriculture worldwide [35–37]. This stress condition leads to huge reductions in crop yields mainly derived from a series of morpho-physiological changes such as reduction in shoot growth [38], decreases in photosynthesis and transpiration rates as a direct consequence of abscisic acid (ABA)-mediated leaf stomata closure [36, 37] as well as changes in signalling pathways [36] and transcriptional and posttranscriptional regulation of several stress-related genes [39, 40]. In addition, plant metabolism is also readjusted under drought stress conditions through the accumulation of osmolytes or compatible solutes [41, 42]. These small molecules can accumulate at high concentrations in the cell without inhibiting cellular metabolism, and comprise, for example, soluble sugars and sugar alcohols such as glucose, sucrose and mannitol; the raffinose family oligosaccharides (RFOs) such as raffinose, stachyose and verbascose, amino acids and polyamines. Because of this osmolyte accumulation, a decrease in the osmotic potential of the cell is observed and the turgor pressure is maintained as the cell uptakes water, thereby help in stabilising membranes, enzymes and proteins, or maintaining cell turgor by osmotic adjustment. In addition, osmolyte accumulation also confers protection against oxidative damage by decreasing the levels of reactive oxygen species (ROS), which in turn, helps re-establish cellular redox balance. Consequently, osmotic adjustment is commonly recognised as an effective factor of drought tolerance in several plants to enable water uptake and the maintenance of plant metabolic activity, hence, growth and productivity

as the water potential decreases [36, 37]. Drought stress has been widely reported to increase the production of ROS in different cellular compartments (i.e., oxidative stress) [43]. However, this oxidative stress has shown to lead to the formation of specific peptides that might counterbalance the accumulation of ROS upon abiotic stress conditions [44]. Nevertheless, ROS species are known to interact with proteins, lipids and DNA during abiotic stress episodes, and thus impair the normal function of cells [45–47].

Comprehensive omics studies have been reported to investigate plant responses to drought stress [42, 48–50]. An interesting study developed by Gechev and collaborators [51] addressed the molecular mechanisms of desiccation in *Haberlea rhodopensis* through transcriptomics and metabolomics approaches. The complementary use of GC-TOF-MS and LC-MS metabolite analyses revealed significant accumulation in the levels of the soluble sugars sucrose and maltose as wells as of the RFOs stachyose and verbascose in *H. rhodopensis* plants upon dehydration. Furthermore, and together with transcriptomics, these results were associated to *H. rhodopensis* ability to survive under dehydration conditions [51].

A similar comprehensive metabolomics approach was applied to study the resurrection plant *Selaginella lepidophylla* [52]. Metabolite profiles from ultra-high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) and GC-MS analysis revealed an accumulation of metabolites involved in the glycolytic pathway (glucose-6-phosphate, fructose-6-phosphate and pyruvate) as well as in the TCA cycle (2-oxoglutarate, succinate, fumarate and oxaloacetate) in hydrated *S. lepidophylla* plants. In parallel, the accumulation of the sugar alcohols sorbitol, myo-inositol and mannitol was related to the desiccation mechanisms developed by resurrection plants, which involve water uptake or loss during the rehydration/dehydration cycle [52].The moderate long-term drought stress effects was investigated in 21 rice cultivars (*Oryza sativa* L. ssp. indica and japonica) through physiological, gene expression and GC-TOF-MS metabolite profiling analysis [53]. Overall, this comprehensive study revealed that in rice, drought conditions induce an accumulation of spermine, thereby leading to a coordinated adjustment of polyamine metabolism which is in agreement with an osmoprotectant role of this metabolite under drought stress [53].

Meyer and co-workers [54] analysed at transcriptional, physiological and metabolite levels the responses to soil drying of the perennial C4 grass and biofuel crop, *Panicum virgatum* L. (switchgrass). In this study, genes associated with C4 photosynthesis were down-regulated during drought, while C4 metabolic intermediates have shown to accumulate. GC-TOF-MS data revealed that the abundance of 13 primary metabolites was significantly affected by the drought treatment and that most of these compounds also accumulated amino acids (>32-fold), monosaccharides (>14-fold) and organic acids (>four-fold) [54]).

GC-TOF-MS metabolite profiling in the leaves and roots of two barley (*Hordeum vulgare* L.) genotypes, with contrasting drought tolerance, revealed approximately 100 drought stress-responsive metabolites with amino acids being the most affected metabolite class. Together with proteomics data, this study indicated that the proteins and metabolites that have shown to accumulate in the susceptible variety also revealed elevated constitutive accumulation levels in the drought-resistant line. Moreover, the accumulation of several carbohydrates was affected in tissues of both genotypes subjected to drought [55].

In sunflower (*Helianthus annuus* L.), molecular mechanisms to drought tolerance were recently addressed through the characterisation and integration of transcriptional and metabolic data. GC-TOF-MS analysis allowed detecting 54 primary metabolites, including different amino acids, organic acids, sugars and sugar alcohols. This analysis revealed that most of the amino acids showed lower levels under drought with exception to proline, tyramine, glycine, malonate and γ-aminobutyrate (GABA), which accumulated upon drought conditions. On the other hand, glycolysis and tricarboxylic acid cycle (TCA) metabolites as well as all the detected carbohydrates showed higher levels under drought conditions. Overall, these results indicated the putative role of these metabolites during stress response in sunflower [56].

Another interesting study investigated osmoadaptation to drought stress in leaves and roots of cowpea (*Vigna unguiculata* L. Walp.) through analysis of photosynthetic traits, water homoeostasis, inorganic ions and primary and secondary metabolites. In this study, physiological and metabolite changes were shown to develop in parallel while drought/recovery responses revealed a progressive acclimation of the cowpea plant to stress. GC-TOF-MS analysis and subsequent multifactorial analyses indicated allocation of high quantities of amino acids, sugars and proanthocyanidins into roots, which were linked to their role in growth and initial stress perception. From the 88 metabolites detected, proline, galactinol and a quercetin derivative, were those that most responded to drought. In addition, these metabolites accumulated differently in roots, but similarly in leaves, suggesting a more conservative strategy to cope with drought in the aerial parts of cowpea plants [57].

3.2. Metabolite responses to salt stress

Soil salinity significantly reduces crop yields, being considered a global problem that affects approximately 20% of irrigated land [58]. The effects of salt stress in plants occur in two different sequential stages. In a first stage, the plant perceives osmotic stress, which reduces the plant's ability to uptake water, decreases cell turgor and leads to the accumulation of ROS in the cells. Subsequently, a second stage is initiated by an over accumulation of Na^+ and Cl^- ions that severely affect key plant physiological processes including photosynthesis, plasma membrane stability and cellular metabolism [59]. Consequently, plant growth and fertility are reduced, and premature senescence occurs [59, 60]. Plant susceptibility or tolerance to salt stress strongly depends on the mechanisms used by the plant to detoxify ROS species within the cells and exclude Na^+ ions from the roots or to compartmentalise these ions in the vacuoles [59, 61]. To cope with salt stress, plants adjust their metabolic status, and although this metabolic adjustment widely differs among salt-tolerant species, several common salt-stress metabolite responses are found within the plant kingdom [62, 63].

According to their salt tolerance, plants are usually divided in glycophytes (salt-sensitive) and halophytes (salt-tolerant). For glycophytic plants, there is an increasing evidence that amino acids, sugars, sugar alcohols and tricarboxylic acid (TCA)-cycle intermediates, form the core of metabolite adjustments to salinity stress [24, 31, 62]. On the other hand, for halophytic or extremophile plants, the pre-accumulation and differential response of osmoprotectant metabolites varies among plant species. Interestingly, a comparative study using both salt-sensitive and salt-tolerant Lotus species has demonstrated that around 50% of all metabolites

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have a comparable response to salinity [64]. A similar scenario was observed for *Arabidopsis thaliana* (salt-sensitive) and its distant relative *Thellungiella halophila* (salt-tolerant), both accumulating proline and soluble sugars (fructose, glucose, sucrose and raffinose) [65].

Among crops, an interesting study on barley (*Hordeum vulgare* L.) cultivars that differed in salt-stress tolerance were analysed for their metabolite response to long-term salt stress [66]. While the most tolerant cultivar Sahara showed elevated levels of hexose phosphates and TCA cycle intermediates, the levels of these metabolites remained unaffected during salinity stress in the less-tolerant cultivar Clipper [66]. In another study, wild barley showed to be more salt-tolerant than cultivated barley by accumulating more carbohydrates (sucrose, trehalose and raffinose) and proline in its roots than its cultivated counterpart, therefore demonstrating an improved ability to regulate osmotic stress [67]. Rice represents one of the most-sensitive cereal crops; however, a GC-TOF-MS analysis revealed lower levels of TCA cycle intermediates and other organic acids in the roots of more-tolerant rice cultivars than in those more sensitive. On the other hand, accumulation of amino acids was detected in the salt-tolerant rice cultivars [68].

A modern metabolomics approach based on two complementary highly sensitive approaches, namely GC- and LC-coupled to a triple quadrupole mass spectrometer (GC-QqQ-MS and LC-QqQ-MS), was applied for the quantitative profiling of a wide range of metabolites from two chickpea (*Cicer arietinum* L.) cultivars with contrasting responses to salt stress. While the GC-QqQ-MS metabolite profiling approach allowed to quantitatively analyse 48 primary metabolites, ranging from sugars and sugar phosphates to organic acids, the LC-QqQ-MS approach allowed to quantitatively measure 28 biogenic amines and amino acids. Furthermore, this complementary approach indicated that the metabolic differences between the two contrasting cultivars relied on metabolites involved in carbon metabolism, TCA cycle as well as amino acid metabolism [69]. A better elucidation of the physiological and biochemical processes of a salt-resistant maize (*Zea mays* L.) hybrid was achieved with GC-TOF-MS metabolite profiling analysis. By comparing a salt-sensitive and a salt-resistant maize hybrid, Richter and co-workers [70] could observe the accumulation of neutral sugars (glucose, fructose and sucrose) in the leaves of the salt-sensitive hybrid and regard these metabolites accumulation as a salt-resistance adaptation. In addition, both hybrids showed a strong decrease in the levels of TCA cycle intermediates [70].

Actinorhizal plants are a group of perennial dicotyledonous angiosperms. These plants are not only of economic importance (production of wood and derivatives), but are also highly resilient to extreme environments. *Casuarina glauca*, the model actinorhizal plant, is characterised by its ability to establish symbiosis with nitrogen-fixing *Frankia* bacteria and can thrive under extreme salinity conditions [71, 72]. However, until now, only few reports investigating the mechanisms underlying salt stress tolerance in actinorhizal plants are available, and most of these studies are not broad enough to grasp the complexity of the response. To better understand *C. glauca* ability to tolerate high levels of salinity, Jorge and collaborators [74] have pioneered a metabolomics study to investigate the impact of salt stress in *C. glauca* nodulated (NOD+) and non-nodulated (KNO_3+) plants subjected to different salinity levels (0 control, 200, 400 and 600 mM [NaCl]) [73]. GC-TOF-MS metabolite profiling data revealed major metabolite divergences in amino acid metabolism in both plant groups (NOD+ and KNO_3+).

Subsequent multivariate statistical analysis allowed concluding that modifications in the metabolite levels of neutral sugars, proline and ornithine revealed to be central in conferring tolerance to high levels of salinity in *C. glauca.* Furthermore, the same study also concluded that the main differences observed in the metabolite pool between NOD+ and KNO_3+ plants not only rely on the impact of the salt stress itself [73], but also on the disruption of the symbiotic activity of *C. glauca* NOD+ plants at early salt stress exposure (i.e., 200 mM [NaCl]) [74].

3.3. Metabolite responses to heat stress

Heat stress is often defined as the rise in temperature beyond a threshold level (usually 10–15°C) above ambient temperature, for an enough period of time, to cause irreversible damage to plant growth and development. The impact of heat stress depends not only on the temperature intensity but also on its duration and rate of increase [75, 76].

When a plant perceives exposure to heat stress, a series of cellular and molecular responses are known to be initiated, such as increased fluidity of lipid membranes, inactivation of key enzymes in some organelles (chloroplasts and mitochondria) and protein denaturation and aggregation. The ability of some plants to grow, develop and give profit under these circumstances is defined as heat tolerance. In plants, the heat stress response (HSR) pathway has been extensively studied [77–79]; however, a more comprehensive understanding of this pathway remains unclear [76].

Heat tolerance has been widely reported in the literature as being mediated by the synthesis of stress-related proteins, also known as heat shock proteins (HSPs) [77, 80]. This class of proteins has shown to confer heat tolerance by reducing the impact of high temperatures in photosynthesis, in carbon assimilate partitioning, in water and nutrient use efficiency as well as in keeping membrane stability [81–83]. General plant cellular and molecular responses to heat stress have been thoroughly reviewed elsewhere [75, 76, 79, 84, 85].

Metabolomics studies on plants subjected to heat stress have reported the accumulation of osmolytes, namely soluble sugars, glycine-betaine and proline [86]. In addition, high temperatures have been reported to disrupt sugar metabolism and proline transport during male reproductive development in tomato (*Solanum lycopersicum* L.) [87].

Du and co-workers [88] applied a GC-MS metabolite profiling approach to identify metabolites associated with differential heat tolerance between two grass species, namely C4 bermudagrass and C3 Kentucky bluegrass [88]. In both grass species, 36 heat stress-responsive metabolites were identified, ranging from organic and amino acids to sugars and sugar alcohols. However, most of these metabolites showed higher accumulation in bermudagrass when compared with Kentucky bluegrass. Among the differentially accumulated metabolites, this study reported seven sugars (sucrose, fructose, galactose, floridoside, melibiose, maltose and xylose), a sugar alcohol (inositol), six organic acids (malic acid, citric acid, threonic acid, galacturonic acid, isocitric acid and methyl malonic acid) and nine amino acids (asparagine, alanine, valine, threonine, GABA, isoleucine, glycine, lysine and methionine) [88].

Using a similar GC-MS metabolic profiling approach, Li and co-workers [89] investigated whether increased GABA levels could improve heat tolerance in cool-season creeping bentgrass

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(*Agrostis Stolonifera* L.) [89]. Upon exogenous application of GABA, metabolite profiling data revealed an accumulation in the levels of six amino acids (glutamic acid, aspartic acid, alanine, threonine, serine and valine), five organic acids (aconitic acid, malic acid, succinic acid, oxalic acid and threonic acid), five sugars (sucrose, fructose, glucose, galactose and maltose) and two sugar alcohols (mannitol and myo-inositol). Together with physiological measurements, this study suggested that the GABA-induced heat tolerance in creeping bentgrass might result from three main factors (i) balance of photosynthesis and transpiration, (ii) improvement of the ascorbate-glutathione cycle and (ii) maintenance of osmotic adjustment. Furthermore, an increase in the levels of metabolites involved in the GABA shunt (glutamic acid, GABA and alanine) was suggested to act as an intermediate supplier to feed the TCA cycle during a long-term heat stress, thereby maintaining metabolic homeostasis [89].

4. Plant metabolite responses to abiotic stress combinations

Plant abiotic stress studies typically deal with the comparison of a few genotypes (tolerant versus sensitive species) grown under controlled conditions, followed by the analysis and identification of differential responses to the imposed stress. Yet, these conditions are unlikely to reproduce field conditions in which a range of abiotic stresses is likely to occur simultaneously. Abiotic stress combinations, such as those involving drought and salinity, salinity and heat as well as drought and extreme temperature or high light intensity are the most commonly reported stress combinations in field conditions [17, 90]. Pioneering abiotic stress combination studies, that involved drought and heat stress, were performed in tobacco (*Nicotiana tabacum* L.) and in the model plant *A. thaliana*. These studies revealed that the molecular responses to this stress combination are unique and should not be regarded as the sum of the responses from each individually applied stress [17, 91, 92]. Afterwards, significant studies have been performed to elucidate the plant molecular responses to several abiotic stress combinations that include drought, salt, extreme temperatures, heavy metals, UV-B, high light, ozone, CO_2, soil compaction and biotic stresses (e.g., pathogen attack) [17, 93]. Likewise, these studies also reported that each stress combination requires specific plant molecular responses. Among them, specific physiological responses as well as specific regulatory transcripts, proteins and metabolites were found for each stress combination under study. Having said this, plant responses to combined stresses require an orchestration of specific metabolic and signalling responses such as antioxidant mechanisms or the synthesis of osmolytes [90, 92, 94–98].

In 2006, Mittler [16] developed an intuitive diagram denominated "*Stress Matrix*" in which the result of a positive and/or negative interaction between two different stress combinations on plant growth, yield and physiological traits can be easily described [16]. Since then, this matrix has been updated several times [17, 93, 99] (**Figure 2**). According to **Figure 2**, most abiotic stress combination studies include drought or salinity as one of the main stress conditions. Stress combinations between drought and heat, salinity and heat, ozone and salinity, ozone and heat, nutrient stress and drought, nutrient stress and salinity (to name a few) were reported to have a higher negative impact on plant development than when each different stress component is applied individually. On the other hand, combinations of drought and ozone, high CO_2 with ozone, salt

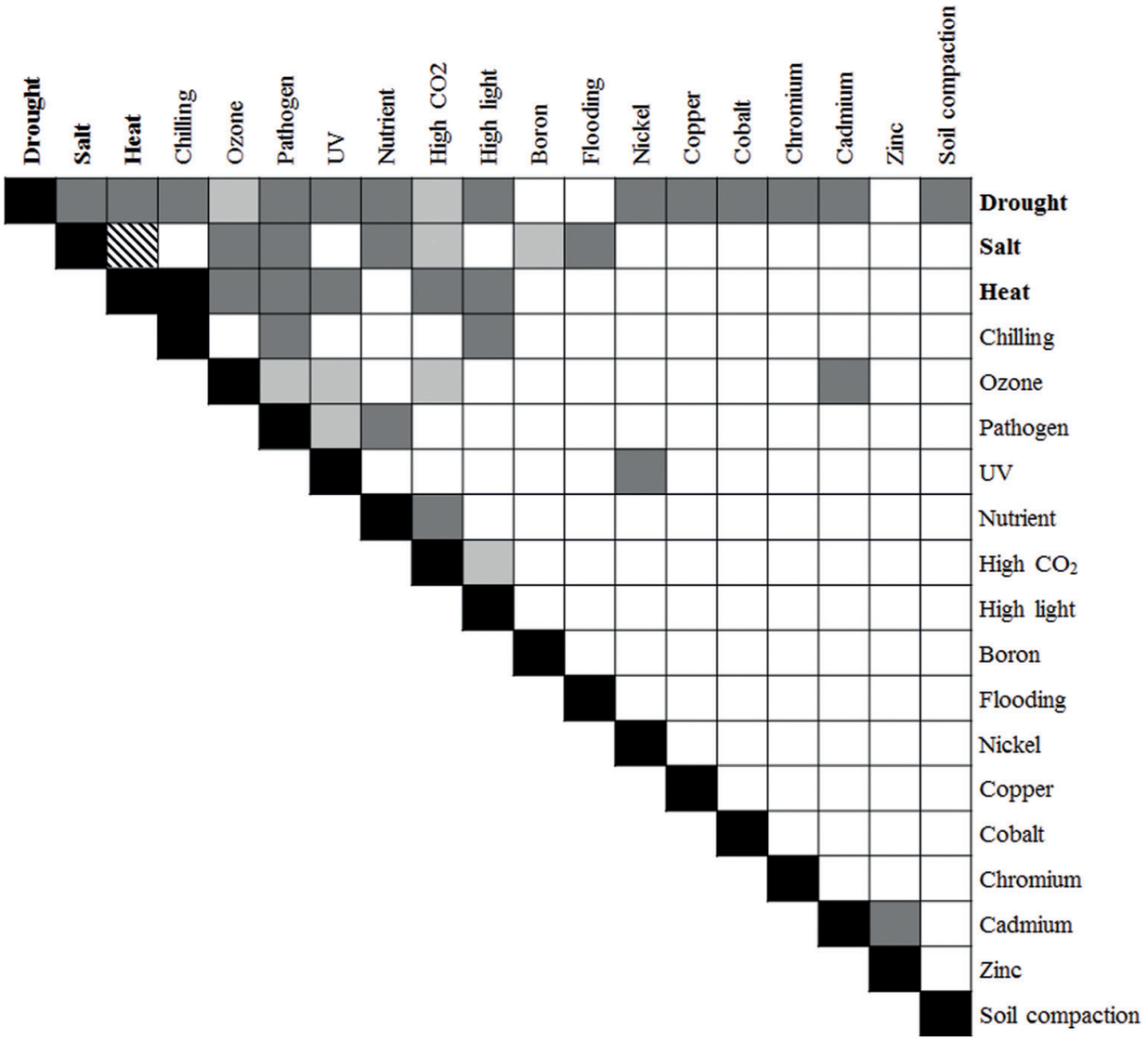

Figure 2. Intuitive "*Stress Matrix*" showing the result of a positive (light grey) and/or negative (dark grey) interaction between two different stress combinations on plant growth, yield and physiological traits. Striped-pattern square indicates a not well-studied species specific-interaction (might be positive and/or negative) (adapted from [16, 17, 97, 103]).

or high light were shown to have a favourable effect on plants as compared to when each different stress component is applied individually [17, 99]. Interestingly, the combination of salinity and heat stress has shown to provide both positive and negative interactions. These conflicting results suggest that the positive or negative effects of a stress combination could be dependent on the plant genotype, species and/or timing and intensity of the different stresses involved. Considering the increased number of heat waves and rising seawater levels expected for the next decades [2], the study of plant metabolite responses to salt and heat stress in a wide range of species is therefore predicted to become increasingly relevant in the current climate change context.

4.1. Metabolite responses to combined drought and heat stress

The effect of drought and heat stress on plant growth and development is currently the most well-studied abiotic stress combination [16, 17, 90], mainly because these two environmental-stress

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factors are the most representative in the field. In addition, they are the primary environmental stresses that determine the distribution and productivity of plants [91, 100]. Following the pioneering studies of the effects of combined drought and heat stress in tobacco and *A. thaliana* [91], many similar studies have been carried out in several other plant species and crops [16, 17, 90, 93, 101]. One interesting study is that of Obata and collaborators [102] who aimed at dissecting the metabolite responses induced by drought, heat and the combination of both stresses in 10 tropical maize hybrids. Through the integration of physiological and metabolomics data, this study identified promising metabolite marker candidates [102]. Under drought stress, GC-TOF-MS analysis of maize leaves revealed the accumulation of several amino acids (isoleucine, valine, threonine, 4-aminobutanoate, glycine and serine) as well as the accumulation of the sugar alcohol *myo*-inositol. On the other hand, when both drought and heat stress were combined, metabolite responses could be predicted from the sum of individual stresses as only a few specific responses could be observed [102].

Metabolite changes under this stress combination were also assessed in the fleshy herbaceous plant Purslane (*Portulaca oleracea* L.) [103]. In total, GC-TOF-MS analysis allowed detecting 37 primary metabolites. Of these, fructose, galactose and xylitol were only detected in control plants; alanine, sorbose, glucose and heptulose were only detected in drought stress-treated plants; glycine, threonine and asparagine were only detected in heat stress-treated plants, while isoleucine and phenylalanine were only found in combined stress-treated plants. On the other hand, propionic acid, gluconic acid, mannose and urea were detected in both individual and combined stress-treated plants. Overall, this study allowed to conclude that the main strategies adopted by purslane to survive drought, heat, and combined drought and heat stress, involves the accumulation of osmoprotectant metabolites and an increase in the antioxidative system [103].

The impact of combined drought and heat stress has also been evaluated in the crop plant soybean (*Glycine max* L.) through a comprehensive MS-based metabolomics approach comprising LC- and GC-MS analysis [104]. This approach allowed identifying 266 putative metabolites, including primary and secondary metabolites. Subsequent statistical analysis revealed that combined drought and heat stress induced a differentially accumulation of several metabolites in soybean leaves, such as sugars, amino acids and lipids. Moreover, individual stresses (i.e., drought or heat) affected key metabolites involved in different pathways such as glycolysis, TCA cycle, the pentose phosphate pathway and starch biosynthesis. That said, this study demonstrated that sugar and nitrogen metabolism are essential in soybean to cope with drought and heat stress conditions [104].

4.2. Metabolite responses to combined drought and salt stress

With increasing earth surface temperatures, it is very likely that regions of high surface salinity, where evaporation dominates, will become more saline [2]. Therefore, it is of great interest to study plant's physiological and metabolite responses to harsh environments where drought and salt stress are occurring simultaneously. However, only a few studies under this context have been performed [105–108]. Among them, only one study addressed maize metabolite responses induced by a combination of drought and salt stress [107]. Indeed, under its natural habitat of irrigated and dry land agricultural lands, maize is exposed to the combined stresses of water deficiency and soil salinity [107]. ^{1}H NMR-based metabolomics analysis of maize leaves revealed that metabolite responses of drought and salt stress differed from those

caused by drought and salt stress applied individually. Additionally, subsequent multivariate statistical analysis allowed identifying those metabolites that specifically responded to the combined stress, namely two TCA cycle intermediates (citrate and fumarate) and four amino acids (the branched chain amino acids—valine, leucine and isoleucine, and the aromatic amino acid—phenylalanine) [107].

4.3. Metabolite responses to combined salt and heat stress

Up to date, studies on the combined effects of salt and heat stress in plants have revealed both positive and negative interactions on plant growth, yield and physiological traits (**Figure 2**). In wheat, the combination of salt and heat stress enhanced the transpiration rate, which in turn, was already induced by heat stress itself. On the other hand, this stress combination also promoted a higher uptake of Na^+ ions by the plant [109, 110].

The effects of the combination of salt and heat stress were evaluated in tomato plants (*Solanum lycopersicum* cv. Optima) [111]. This stress combination was observed to induce a specific response by the plants through the accumulation in the levels of glycine betaine and trehalose, both well-known for their osmoprotectant roles. The accumulation of glycine betaine and trehalose was associated to the maintenance of a lower Na^+:K^+ ratio, thereby leading to a better performance of the cell water status and photosynthesis when compared to the salt stress alone [111].

To the best of our knowledge, metabolomics studies aiming at dissecting metabolite responses induced by salt and heat stress are scarce, highlighting the need for further research in this area.

5. Concluding remarks

Climate change disturbs a number of variables that determine how much plants can grow and develop. Extreme temperatures, elevated CO_2 together with a decrease in water availability and changes to soil conditions will essentially make it more challenging for plants to thrive. Overall, climate change is expected to decline the growth and development of plants, particularly with reference to agricultural systems. Declining plant growth also dramatically changes the habitats that are necessary for many species to survive. Undoubtedly, under the current threat of climate change, it is urgent to address the molecular and biochemical mechanisms that underlie plant responses to several abiotic stresses and combinations thereof. However, a complete understanding of plant responses to climate change is best obtained if data is integrated at several levels, including morpho-physiological and developmental studies as well as molecular studies that comprise the so-called *omics* technologies. Up to now, metabolomics studies have already provided a promising basis for facilitating our understanding of the plant's flexibility to reconfigure central metabolic pathways (i.e., carbon, nitrogen and energy metabolism) as well as the degree by which plants tolerate and/or are susceptible to a climate change scenario. Nevertheless, more research efforts are crucial for a more comprehensive

analysis of the impact of combined stresses in plants. Researchers must regard simultaneous multiple climate change factors, which sum will play a key negative influence on global agriculture, as a new state of stress in which the exposed plant might require differential responses from those induced by a stress alone. Further research in this area is therefore critical.

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